

**PROTOCOL**

**AOAC Use-Dilution Method**

**Test Organism:**

*Listeria monocytogenes* (ATCC 19117)

**PROTOCOL NUMBER**

KIK02012213.UD.5

EXACT COPY  
INITIALS JS DATE 3-5-13

**PREPARED FOR**

KIK Custom Products  
909 Magnolia Avenue  
Auburndale, FL 33823

**PERFORMING LABORATORY**

ATS Labs  
1285 Corporate Center Drive, Suite 110  
Eagan, MN 55121

**PREPARED BY**

Gracia Schroeder, B.S.  
Microbiologist

**DATE**

January 22, 2013

**PROPRIETARY INFORMATION**

THIS DOCUMENT IS THE PROPERTY OF AND CONTAINS PROPRIETARY INFORMATION OF ATS LABS. NEITHER THIS DOCUMENT, NOR INFORMATION CONTAINED HEREIN IS TO BE REPRODUCED OR DISCLOSED TO OTHERS, IN WHOLE OR IN PART, NOR USED FOR ANY PURPOSE OTHER THAN THE PERFORMANCE OF THIS WORK ON BEHALF OF THE SPONSOR, WITHOUT PRIOR WRITTEN PERMISSION OF ATS LABS.

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### AOAC Use-Dilution Method

**SPONSOR:** KIK Custom Products  
909 Magnolia Avenue  
Auburndale, FL 33823

**TEST FACILITY:** ATS Labs  
1285 Corporate Center Drive, Suite 110  
Eagan, MN 55121

#### **PURPOSE**

The purpose of this study is to determine the effectiveness of the Sponsor's product as a disinfectant for hard surfaces following the AOAC Use-Dilution Method. This method is in compliance with the requirements of the following: The U. S. Environmental Protection Agency (EPA).

#### **TEST SUBSTANCE CHARACTERIZATION**

Test substance characterization as to content, stability, etc., (40 CFR, Part 160; Subpart F (160.105)) is the responsibility of the Sponsor. The test substance shall be characterized by the Sponsor prior to the experimental start date of this study. Pertinent information, which may affect the outcome of this study, shall be communicated in writing to the Study Director upon sample submission to ATS Labs.

#### **SCHEDULING AND DISCLAIMER OF WARRANTY**

Experimental start dates are generally scheduled on a first-come/first-serve basis once ATS Labs receives the Sponsor approved/completed protocol, signed fee schedule and corresponding test substance(s). Based on all required materials being received at this time, the proposed experimental start date is January 28, 2013. Verbal results may be given upon completion of the study with a written report to follow on the proposed completion date of February 25, 2013. To expedite scheduling, please be sure all required paperwork and test substance documentation is complete/accurate upon arrival at ATS Labs.

If a test must be repeated, or a portion of it, due to failure by ATS Labs to adhere to specified procedures, it will be repeated free of charge. If a test must be repeated, or a portion of it, due to failure of internal controls, it will be repeated free of charge. "Methods Development" fees shall be assessed, however, if the test substance and/or test system require modifications due to complexity and difficulty of testing.

If the Sponsor requests a repeat test, they will be charged for an additional test.

Neither the name of ATS Labs nor any of its employees are to be used in advertising or other promotion without written consent from ATS Labs.

The Sponsor is responsible for any rejection of the final report by the regulating agencies concerning report format, pagination, etc. To prevent rejection, Sponsor should carefully review the ATS Labs final report and notify ATS Labs of any perceived deficiencies in these areas before submission of the report to the regulatory agency. ATS Labs will make reasonable changes deemed necessary by the Sponsor, without altering the technical data.

#### **JUSTIFICATION FOR SELECTION OF THE TEST SYSTEM**

Regulatory Agencies require that a specific organism claim for a test substance intended for use on hard surfaces be supported by appropriate scientific data demonstrating the efficacy of the test substance against the claimed organism. This is accomplished in the laboratory by treating the target organism with the test substance under conditions which simulate as closely as possible the actual conditions under which the test substance is designed to be used. For products intended for use on hard surfaces (dry, inanimate environmental surfaces), a carrier method is used in the generation of the supporting data. The experimental design in this protocol meets these requirements.

Template: 210-11A

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#### TEST PRINCIPLE

A film of organism cells dried on a surface of stainless steel carriers is exposed to the test substance for a specified exposure time. After exposure, the carriers are transferred to vessels containing neutralizing subculture media and assayed for survivors. Appropriate culture purity, sterility, viability, carrier population and neutralization confirmation controls are performed. The current version of Standard Operating Procedure CGT-4400 reflects the methods which shall be used in this study.

#### TEST METHOD

| Test Organism                 | ATCC # | Growth Medium              | Incubation Parameters |
|-------------------------------|--------|----------------------------|-----------------------|
| <i>Listeria monocytogenes</i> | 19117  | Brain Heart Infusion broth | 35-37°C, aerobic      |

The test organism to be used in this study was obtained from the American Type Culture Collection (ATCC), Manassas, VA.

Subculture Medium: Neutralizing broth appropriate for the test substance.

#### Carriers

Carriers will be screened according to AOAC Official Method of Analysis and any carrier positive for growth will be discarded. Only penicylinders showing no growth may be used. Stainless steel penicylinders will be pre-soaked overnight in 1N NaOH, washed in water until neutral and autoclaved in deionized water. Carriers shall be used within three months of sterilization.

#### Preparation of Test Organism

A loopful of stock slant culture or 10 µL of a thawed cryovial of stock organism broth culture will be used to inoculate the initial 10 mL tube of growth medium.

Mix and incubate the initial culture for 24±2 hours at 35-37°C. Following incubation and without vortex mixing the initial culture, transfer 10 µL of culture to sufficient 10 mL tubes of culture medium (daily transfer #1). Up to four additional daily transfers may be prepared. The final test culture will be prepared using culture from daily transfer # 1-5. Incubate the final test culture for 48-54 hours at 35-37°C.

The test culture will be vortex mixed for 3 to 4 seconds and allowed to stand for ≥10 minutes prior to use. After this time, the upper portion of the culture will be removed, leaving behind any clumps or debris and will be pooled in a sterile vessel. The culture may be diluted. Applicable culture dilutions shall be performed using sterile growth medium and shall not exceed a 1:2 dilution (one part test culture to one part sterile growth medium). An organic soil load will be added to the test culture per Sponsor's request. The final test culture will be mixed thoroughly prior to use.

#### Contamination of Carriers

The culture will be transferred to the penicylinders and the carriers will be immersed for 15±2 minutes in a prepared suspension at a ratio of one carrier per one mL of culture. A maximum of 100 carriers will be inoculated per vessel and each vessel inoculated may be considered a part of one total inoculation run per organism. The inoculated carriers will be transferred to sterile Petri dishes matted with filter paper. No more than twelve carriers will be placed in each Petri dish. The carriers will be dried for 40±2 minutes at 25-30°C and ≥60% humidity.

#### Preparation of Test Substance

The test substance(s) to be assayed will be used as directed by the Sponsor. If a dilution of the test substance is requested by the Sponsor, the diluted test substance(s) shall be used within three hours of preparation. Ten (10) mL of the test substance at its use-dilution will be aliquotted into the required number of sterile 25 x 150 mm or 25 x 100 mm tubes. The tubes will be placed into a waterbath at the specified exposure temperature, and allowed to equilibrate for ≥10 minutes prior to testing.

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#### **Exposure Conditions**

Each contaminated and dried carrier will be placed into a separate tube containing 10 mL of the test substance at its use-dilution for the desired exposure time and temperature. Immediately after placing each test carrier in the test tube, swirl the tube using approximately 2-3 gentle rotations to release any air bubbles trapped in or on the carrier.

#### **Test System Recovery**

Following the Sponsor specified exposure time, each medicated carrier will be transferred by wire hook at staggered intervals to 10-20 mL of primary neutralizing subculture medium. To accomplish this, the carrier is removed from the disinfectant tube with a sterile hook, tapped against the interior sides of the tube to remove the excess disinfectant and transferred into the subculture tube. Avoid tapping the carrier against the approximate upper third of the tube. If secondary neutralization is requested by the Sponsor or deemed necessary due to test substance active and/or concentration, carriers will be transferred into individual secondary subculture tubes containing 10-20 mL of neutralizing broth beginning approximately 25-60 minutes after subculture of the carrier into the primary neutralizing subculture medium.

#### **Incubation and Observation**

All subculture vessels and control plates are incubated for  $48 \pm 2$  hours at  $35-37^{\circ}\text{C}$ .

Following incubation, the subcultures will be visually examined for growth. If necessary, the subcultures may be placed at  $2-8^{\circ}\text{C}$  for up to three days prior to examination.

Representative subculture tubes showing growth will be subcultured, stained and/or biochemically assayed to confirm or rule out the presence of the test organism.

#### **STUDY CONTROLS**

##### **Purity Control**

A "streak plate for isolation" will be performed on the organism culture and following incubation examined in order to confirm the presence of a pure culture. The acceptance criterion for this study control is a pure culture demonstrating colony morphology typical of the test organism.

##### **Organic Soil Sterility Control**

The serum used for soil load will be cultured, incubated, and visually examined for lack of growth. The acceptance criterion for this study control is lack of growth.

##### **Carrier Sterility Control**

A representative uninoculated carrier will be added to the neutralizing subculture medium. The subculture medium containing the carrier will be incubated and examined for growth. The acceptance criterion for this study control is lack of growth.

##### **Neutralizing Subculture Medium Sterility Control**

A representative sample of uninoculated neutralizing subculture medium will be incubated and visually examined. The acceptance criterion for this study control is lack of growth.

##### **Viability Control**

Two representative inoculated carriers will each be added to individual vessels containing the subculture medium. If secondary subcultures are performed, one carrier will be placed in the primary subculture medium and one carrier will be placed in the secondary subculture medium. The vessels containing each carrier will be incubated and visually examined for growth. The acceptance criterion for this study control is growth in the final subculture medium, minimally.

#### Neutralization Confirmation Control

The neutralization of the test substance will be confirmed by exposing at least one sterile carrier to the test substance and transferring the carrier to primary subcultures containing 10-20 mL of neutralizing subculture medium as in the test. If performed in the test procedure, each carrier will then be transferred from primary subcultures into individual secondary subcultures beginning approximately 25-60 minutes following the primary transfer. The subcultures (primary and secondary as applicable) will be inoculated with a target of 10-100 colony forming units (CFU) of each test organism, incubated under test conditions and visually examined for the presence of growth. This control will be performed with multiple replicates using different dilutions of the test organism. A standardized spread plate procedure will be run concurrently in order to enumerate the number of CFU actually added. The control result will be reported using data from the most appropriate dilution. NOTE: Only the most concentrated test substance and/or shortest exposure time needs to be evaluated in this control.

The acceptance criterion for this study control is growth in the final subculture broth, minimally, following inoculation with  $\leq 100$  CFU.

#### Carrier Population Control

Two sets of three inoculated carriers (one set prior to testing and one set following testing) for each organism carrier set will be assayed. Each inoculated carrier will be individually subcultured into a tube containing 10 mL of neutralizing subculture medium and sonicated for approximately 1 minute. Following sonication, the contents of the three subcultured carriers will be pooled (30mL). Appropriate serial ten-fold dilutions will be prepared and the duplicate aliquots spread plated on agar plate medium, and incubated. If serial dilutions are not performed and plated immediately following sonication, the vessels may be refrigerated at 2-8°C prior to dilution. Following incubation, the resulting colonies will be enumerated and the CFU per carrier calculated. The individual CFU per carrier set results will be calculated, and the  $\log_{10}$  value of each carrier set determined. The average  $\log_{10}$  value per organism will be calculated. The acceptance criterion for this study control is a minimum average  $\log_{10}$  value of 4.0.

#### PROCEDURE FOR IDENTIFICATION OF THE TEST SYSTEM

ATS Labs maintains Standard Operating Procedures (SOPs) relative to efficacy testing studies. Efficacy testing is performed in strict adherence to these SOPs which have been constructed to cover all aspects of the work including, but not limited to, receipt, log-in, and tracking of biological reagents including test organism strains for purposes of identification, receipt and use of chemical reagents. These procedures are designed to document each step of efficacy testing studies. Appropriate references to medium, batch number, etc. are documented in the raw data collected during the course of each study.

Additionally, each efficacy test is assigned a unique Project Number when the protocol for the study is initiated by the Study Director. This number is used for identification of the test subcultures, etc. during the course of the test. Test subcultures are also labeled with reference to the test organism, experimental start date, and test product. Microscopic and/or macroscopic evaluations of positive subcultures are performed in order to confirm the identity of the test organism. These measures are designed to document the identity of the test system.

#### METHOD FOR CONTROL OF BIAS: NA

## STUDY ACCEPTANCE CRITERIA

### **Test Substance Performance Criteria**

The U.S. EPA efficacy performance requirements for label claims state that the test substance must kill the microorganism on 10 out of the 10 inoculated carriers.

### **Control Acceptance Criteria**

The study controls must perform according to the criteria detailed in the study controls description section. If any control acceptance criteria are not met, the test may be repeated under the current protocol number.

## REPORT

The report will include, but not be limited to, identification of the sample, date received, initiation and completion dates, identification of the organism strains used, description of media and reagents, description of the methods employed, tabulated results and conclusion as it relates to the purpose of the test, and all other items required by 40 CFR Part 160.185.

## PROTOCOL CHANGES

If it becomes necessary to make changes in the approved protocol, the revision and reasons for changes will be documented, reported to the Sponsor and will become a part of the permanent file for that study. Similarly, the Sponsor will be notified as soon as possible whenever an event occurs that may have an effect on the validity of the study.

Standard operating procedures used in this study will be the correct effective revision at the time of the work. Any minor changes to SOPs (for this study) or methods used will be documented in the raw data and approved by the Study Director.

## TEST SUBSTANCE RETENTION

It is the responsibility of the Sponsor to retain a sample of the test substance. All unused test substance will be discarded following study completion unless otherwise indicated by Sponsor.

## RECORD RETENTION

### **Study Specific Documents**

All of the original raw data developed exclusively for this study shall be archived at ATS Labs. These original data include, but are not limited to, the following:

1. All handwritten raw data for control and test substances including, but not limited to notebooks, data forms and calculations.
2. Any protocol amendments/deviation notifications.
3. All measured data used in formulating the final report.
4. Memoranda, specifications, and other study specific correspondence relating to interpretation and evaluation of data, other than those documents contained in the final study report.
5. Original signed protocol.
6. Certified copy of final study report.
7. Study-specific SOP deviations made during the study.

### **Facility Specific Documents**

The following records shall also be archived at ATS Labs. These documents include, but are not limited to, the following:

1. SOPs which pertain to the study conducted.
2. Non study-specific SOP deviations made during the course of this study which may affect the results obtained during this study.
3. Methods which were used or referenced in the study conducted.
4. QA reports for each QA inspection with comments.
5. Facility Records: Temperature Logs (ambient, incubator, etc.), Instrument Logs, Calibration and Maintenance Records.
6. Current curriculum vitae, training records, and job descriptions for all personnel involved in the study.

#### REFERENCES

1. Association of Official Analytical Chemists (AOAC) Official Method 964.02, Testing Disinfectants against *Pseudomonas aeruginosa* - Use-Dilution Method. In Official Methods of Analysis of the AOAC, 2012 Edition.
2. Association of Official Analytical Chemists (AOAC) Official Method 955.15, Testing Disinfectants against *Staphylococcus aureus* - Use-Dilution Method. In Official Methods of Analysis of the AOAC, 2012 Edition.
3. Association of Official Analytical Chemists (AOAC) Official Method 955.14, Testing Disinfectants against *Salmonella enterica* - Use-Dilution Method. In Official Methods of Analysis of the AOAC, 2012 Edition.
4. Association of Official Analytical Chemists (AOAC), 2005. Germicidal and Detergent Sanitizing Action of Disinfectants Method 960.09 [Preparation of Synthetic Hard Water].
5. U.S. Environmental Protection Agency, Office of Chemical Safety and Pollution Prevention, Product Performance Test Guidelines, OCSPP 810.2000: General Considerations for Uses of Antimicrobial Agents, September 4, 2012.
6. U.S. Environmental Protection Agency, Office of Chemical Safety and Pollution Prevention, Product Performance Test Guidelines, OCSPP 810.2200: Disinfectants for Use on Hard Surfaces- Efficacy Data Recommendations, September 4, 2012.

#### DATA ANALYSIS

##### Calculations

Determine the CFU/Carrier set in the Carrier Population Control using all average counts between 0-300 CFU as follows:

$$\text{CFU/carrier} = \frac{[(\text{avg. CFU for } 10^{-x}) + (\text{avg. CFU for } 10^{-y}) + (\text{avg. CFU for } 10^{-z})] \times (\text{Volume of neutralizer})}{[10^{-x} + 10^{-y} + 10^{-z}] \times (\text{Volume plated}) \times (\# \text{ of carriers per set})}$$

where  $10^{-x}$ ,  $10^{-y}$ , and  $10^{-z}$  are example dilutions that may be used

$$\text{Average Log}_{10} \text{ Carrier Population Control} = \frac{\text{Log}_{10} X_1 + \text{Log}_{10} X_2 + \dots + \text{Log}_{10} X_N}{N}$$

Where: X equals CFU/carrier set  
N equals number of control carrier sets

##### Statistical Analysis

None used.

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### STUDY INFORMATION

(All sections must be completed prior to submitting protocol)

Test Substance (Name & Batch Numbers, including ≥60 day old batch - exactly as it should appear on final report):

PURE BRIGHT GERMICIDAL ULTRA BLEACH, 130220955M + 130250719M

Specify ≥60 day old batch: N/A

Expiration Date: Aug 1, 2013

EPA Reg. No. 70271-13

cc Jn 2-14-13

#### Product Description:

- ☐ Quaternary ammonia  
☐ Iodophor  
☒ Sodium hypochlorite

- ☐ Peracetic acid  
☐ Peroxide  
☐ Other

Test Substance Active Concentration (upon submission to ATS Labs):

6%

Neutralization/Subculture Broth:

- ☐   
☒ ATS Labs' Discretion. By checking, the Sponsor authorizes ATS Labs, at their discretion, to perform neutralization confirmation assays at the Sponsor's expense prior to testing to determine the most appropriate neutralizer. (See Fee Schedule).

#### Storage Conditions:

- ☒ Room Temperature  
☐ 2-8°C  
☐ Other

#### Hazards:

- ☐ None known: Use Standard Precautions  
☒ Material Safety Data Sheet, Attached for each product  
☐ As Follows:

#### Product Preparation

- ☐ No dilution required, Use as received (RTU)  
☒ \*Dilution(s) to be tested:  
3/4 cup per gallon defined as 3/4 Cup + 1 Gallon  
(example: 1 oz/gallon) \*\* (amount of test substance) (amount of diluent)  
☐ Deionized Water (Filter or Autoclave Sterilized)  
☒ Tap Water (Filter or Autoclave Sterilized)  
☐ AOAC Synthetic Hard Water: PPM  
☐ Other

\*\* SEE PROTOCOL MODIFICATION

\*Note: An equivalent dilution may be made unless otherwise requested by the Sponsor.

Test Organism: ☒ *Listeria monocytogenes* (ATCC 19117)

Carrier Number: 10 per batch

Exposure Time: 5 Minutes

Exposure Temperature: 20 ± 1 °C

#### Organic Soil Load:

- ☐ Minimum 5% Organic Soil Load (Fetal Bovine Serum)  
☒ No Organic Soil Load Required  
☐ Other

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**TEST SUBSTANCE SHIPMENT STATUS**

- ☐ Has been used in one or more previous studies at ATS Labs.  
☐ Has been shipped to ATS Labs (but has not been used in a previous study).  
Date shipped to ATS Labs: \_\_\_\_\_ Sent via overnight delivery? ☐ Yes ☐ No  
☒ Will be shipped to ATS Labs.  
Date of expected receipt at ATS Labs: FEB. 5, 2013  
☐ Sender (If other than Sponsor): \_\_\_\_\_

**COMPLIANCE**

Study to be performed under EPA Good Laboratory Practice regulations (40 CFR Part 160) and in accordance to standard operating procedures.

- ☒ Yes  
☐ No (Non-GLP Study)

**PROTOCOL MODIFICATIONS**

- ☐ Approved without modification  
☒ Approved with modification


Prior to testing, perform the following per product preparation, per day: Titrate test substance per ATS Labs SOP CGT-0090 to determine starting sodium hypochlorite concentration. Dilute test substance to 66,500-67,000 ppm with sterile tap water and titrate to confirm. Dilute test substance per page 8 of protocol for use in testing.

**PROTOCOL ATTACHMENTS**

Supplemental Information Form Attached - ☐ Yes ☒ No

**APPROVAL SIGNATURES**

**SPONSOR:**

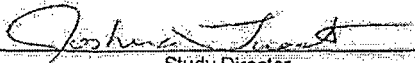
NAME: Mr. Justin Lowe TITLE: Regional QA Manager  
SIGNATURE:  DATE: 1/29/2013  
PHONE: (863) 551-3006 FAX: \_\_\_\_\_ EMAIL: llowe@kikcorp.com

For confidentiality purposes, study information will be released only to the sponsor/representative signing the protocol (above) unless other individuals are specifically authorized in writing to receive study information.

Other individuals authorized to receive information regarding this study: ☐ See Attached

CRISTINA GRIFFIN - CGRIFFIN@DELTA-AC.COM

**ATS Labs:**

NAME: Joshua Luedtke  
Study Director  
SIGNATURE:  DATE: 2-14-13  
Study Director

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